Cell, Vol. 104, 643–646, March 9, 2001, Copyright ©2001 by Cell Press

Minireview

The Control of Mammalian DNA Replication: A Brief History of Space and Timing

Daniel M. Cimbora*§ and Mark Groudine*†‡
*Division of Basic Sciences
Fred Hutchinson Cancer Research Center
Seattle, Washington 98109
†Department of Radiation Oncology
University of Washington School of Medicine
Seattle, Washington 98195

Replication of the eukaryotic genome initiates at specific locations, termed origins, and progresses in a defined temporal order during S phase of the cell cycle. In higher eukaryotes our understanding of how origins are selected, and how replication timing is controlled, is far from complete. Recent experiments using a system that involves the incubation of intact mammalian nuclei in replication-competent *Xenopus* egg extracts have revealed roles for nuclear organization in origin selection, differences in the mechanism of origin specification between mammals and yeast, and an intriguing correlation between the repositioning of chromosomal domains after mitosis and the programming of replicating timing. Interestingly, these experiments have also shown that the specification of origins and programming of replication timing during S phase are independent events that occur during the preceding G1 phase.

Orc Proteins and the Origin Decision Point

Biochemical analyses of known origins in higher eukaryotes have revealed common sequence and structural features such as DNA unwinding elements, binding sites for replication proteins and transcription factors, and sites of attachment to the nuclear matrix/scaffold. Moreover, genetic analyses have demonstrated that a discrete DNA segment containing an origin is necessary, and in some chromosomal contexts sufficient, for origin activity, and that sequences far from an origin can influence origin activity (Aladjem et al., 1998 and Cimbora et al., 2000 and references therein). However, we are still far from understanding *how* such elements specify an initiation site within a chromosomal context. Recent experiments have begun to unravel the biochemical events that lead to origin selection, and suggest that epigenetic factors such as chromatin structure can influence origin choice.

According to current models of replication in yeast, the origin recognition complex (ORC), composed of six conserved Orc proteins, associates with DNA in a sequence-specific manner throughout the cell cycle (references in Mizushima et al., 2000). During G1 phase, ORC recruits other factors including Cdc6 and the Mcm proteins to form a prereplication complex. Initiation of replication is then triggered by the association of additional factors and cyclin-dependent kinase (CDK) activity at the G1/S transition. Upon S phase entry, the prereplication complex is partially disassembled with the release of Cdc6 and Mcm proteins, preventing reinitiation of DNA replication until the next cell cycle. The association of an intact yeast ORC with origins throughout the cell cycle suggests that the first regulated step in prereplication complex assembly in yeast is the association of Cdc6 with ORC.

Because of similarities in structure and function of ORC and other replication proteins, models of metazoan replication are based in large part on the yeast model. However, recent experiments demonstrate that in contrast to yeast, mammalian ORC is not a constitutive chromatin-bound complex, but rather partially disassembles during the cell cycle. The first hint of this came from the observation that the initiation of replication at specific origins in mammalian nuclei is a property acquired during the preceding G1 phase (references in Gilbert, 1998). In this experiment, when nuclei isolated from early G1 phase Chinese hamster ovary (CHO) cells were incubated in *Xenopus* replication extract, the hamster dihydrofolate reductase (DHFR) locus replicated without apparent initiation site preference. In contrast, in nuclei isolated at least 3–4 hr after metaphase, the DHFR locus replicated from the same specific origin (ori-β) used by hamster cells in culture. The point during G1 at which origin specificity is acquired was termed the origin decision point (ODP; Figure 1). Immunodepletion experiments revealed that at the ODP, replication in CHO nuclei ceases to be dependent on *Xenopus* Orc proteins in the extract (Natale et al., 2000), suggesting that the ODP reflects the appearance of functional hamster ORCs. This is not due to differential expression of either of two hamster Orc proteins examined: Orc1 and Orc2 are expressed at similar, constant levels during M and G1. However, in contrast to Orc2, which is stably associated with chromatin during M and G1, the affinity of Orc1 binding to chromatin was found to vary from low during mitosis and early G1 to high in mid-G1. The stable association of Orc1 with chromatin coincides temporally with the ODP, suggesting that origin specification in hamster nuclei is due, at least in part, to the assembly of an intact ORC at origins.

The dynamic behavior of ORC evident in hamster nuclei seems to be a general phenomenon in mammals. In human cells, Orc1 is released from chromatin during a subset of the cell cycle while the bulk of Orc2 remains bound to chromatin (Kreitz et al., 2000), although there are conflicting reports regarding human Orc1 behavior in the literature. In addition, in vivo footprinting reveals the presence of an ORC-like complex bound to the human lamin B2 origin during G1 but not mitosis (Abdurashidova et al., 1998). Furthermore, ORC behavior is not the only difference between mammals and yeast: recent experiments with elutriated human cells demonstrate that a significant fraction of Cdc6 protein associates with chromatin throughout the cell cycle, in contrast to the regulated dissociation of yeast Cdc6 from prereplication complexes during S phase (Mendez and Stillman, 2000). Together, these results suggest a model for mammalian origin specification in which Orc2 and Cdc6 re-

‡To whom correspondence should be addressed (e-mail: markg@fhcrc.org).
§Present address: Myriad Genetics, Inc., Salt Lake City, Utah 84108.

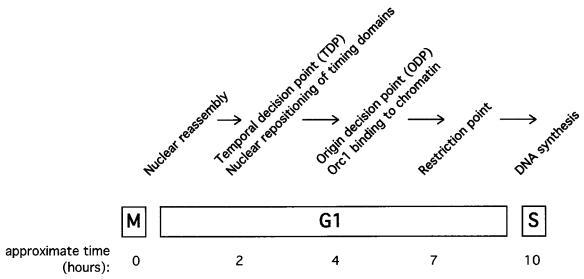

Figure 1. Events during G1 Phase of the Cell Cycle Contribute to Origin Specification and Replication Timing in the Following S Phase

Replication timing is programmed at the temporal decision point (TDP), coincident with repositioning of chromosomal domains following mitosis. Nuclei acquire the ability to recognize specific origins at the origin decision point (ODP), which can be attributed, at least in part, to the binding of Orc1 to origins, generating a functional ORC. The TDP and ODP precede the restriction (R) point, at which entry into S phase becomes independent of growth conditions.

main associated with origins throughout the cell cycle, while Orc1 cycles on and off chromatin (Figure 2). Other Orc proteins may remain associated with Orc2 on chromatin, or may dissociate along with Orc1. The selection of sites for ORC assembly during G1 may be influenced by Cdc6, as it has recently been shown that yeast Cdc6 inhibits nonspecific DNA binding of yeast ORC in vitro (Mizushima et al., 2000). The partial disassembly and reassembly of mammalian ORC during the cell cycle is a potential control step not evident in yeast, providing an opportunity to vary origin choice from one cell cycle to the next. Origin usage in metazoans is dynamic, undergoing changes during embryonic development, upon alteration of gene activity or chromatin structure, and during gametogenesis. Regulating the assembly of an intact ORC at specific chromosomal sites is one mechanism by which these changes in origin use might occur.

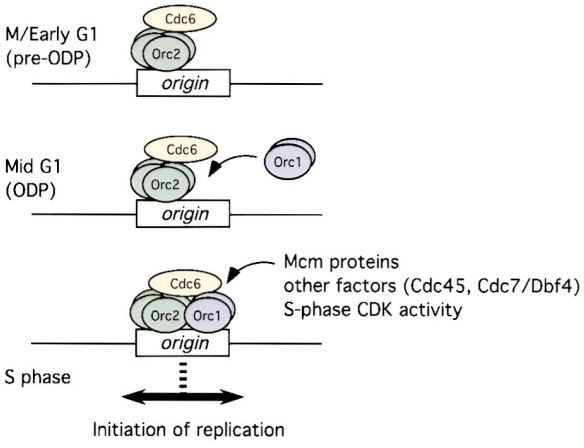

Figure 2. Model for Origin Selection and Prereplication Complex Assembly during G1 in Mammalian Nuclei

Orc2 (and perhaps other Orc proteins) and Cdc6 are bound to chromatin throughout the cell cycle. At the ODP, Orc1 and perhaps other Orcs bind to form an intact ORC. Mcm proteins and additional factors are recruited, and CDK activity triggers replication initiation at S phase.

A Role for the Nuclear Envelope in Origin Selection

Disrupting nuclear integrity has been shown to abolish origin specificity in late G1 (post-ODP) CHO nuclei: permeabilization in such a way that nuclei are unable to exclude large molecules results in replication of the DHFR locus without a preferred initiation site (references in Gilbert, 1998). This likely reflects a requirement for an intact nuclear envelope in restricting the access of *Xenopus* factors (perhaps ORC, which is highly abundant in egg extracts) to chromatin. Studies in *Xenopus* embryos at the mid-blastula transition (MBT) reveal a progressive shift from promiscuous, high frequency origins to widely spaced, defined origins as the number of nuclei increases. The necessity for regulating the intranuclear concentration of critical factors is revealed by experiments showing a requirement for the nuclear envelope and a direct relationship between the origin spacing and the concentration of nuclei in *Xenopus* extracts (references in Walter et al., 1998). Similarly, the concentration of CHO nuclei in *Xenopus* extract has an effect on the preference for DHFR ori-β, with maximal use of ori-β at a concentration of nuclei similar to that found at the MBT (Dimitrova and Gilbert, 1998). More recently it has been shown that a single round of replication can occur in vitro in the absence of nuclear structure by the sequential addition of *Xenopus* cytoplasmic extract and a nucleoplasmic extract of sufficiently high concentration (Walter et al., 1998). Taken together, these results suggest that the role of the nuclear envelope is to maintain a low enough concentration of factors (perhaps ORC) in the nucleus to prevent nonspecific binding to chromatin, while maintaining a sufficiently high concentration of other nuclear factors to ensure efficient activation of prereplication complexes and their disassembly from chromatin after initiation.

Chromatin Structure and Origin Choice

Chromatin structure appears to play a role in origin selection. The transition to specific origins at the *Xenopus* MBT is accompanied by a variety of changes including the onset of zygotic transcription, changes in chromatin structure, and changes in the attachment of chromatin to the nucleoskeleton. Although it has long been observed that nascent DNA is associated with the nucleoskeleton and that matrix/scaffold attachment sites are often found near replication origins, changes in nucleoskeletal attachment have been ruled out as a determinant of origin specificity at the MBT (Maric and Hyrien, 1998). In contrast, altered histone H4 acetylation and the incorporation of histone H1 into chromatin at the MBT suggest a relationship between chromatin structure and origin selection; this relationship is supported by recent experiments demonstrating that histone H1 has a direct inhibitory effect on the assembly of prereplication complexes on *Xenopus* sperm chromatin (Lu et al., 1998 and references therein). Furthermore, when condensed metaphase chromosomes from hamster cells are added to *Xenopus* extracts, the DHFR locus is replicated from a novel origin, and activity of this novel origin is dependent on topoisomerase II–mediated chromatin condensation (references in Gilbert, 1998). Likewise, in yeast, an origin that is not normally active becomes active when the silent chromatin component Sir3 is mutated (Stevenson and Gottschling, 1999). Covalent modifications of DNA also appear to play a role

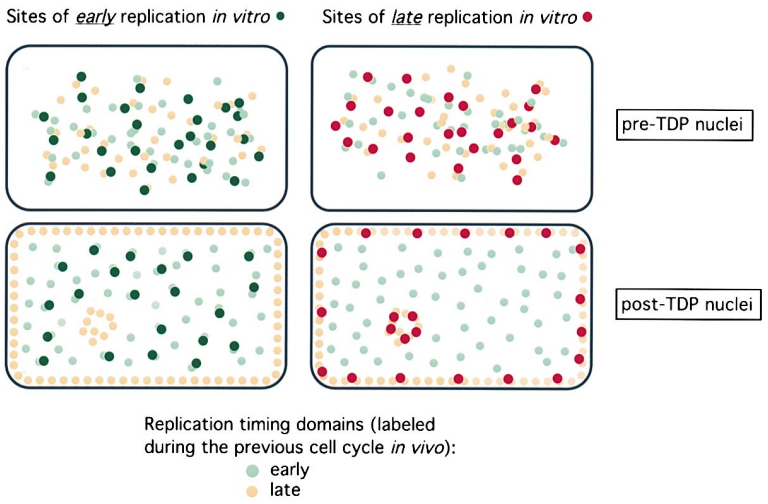

Figure 3. Once the TDP Is Reached, Chromosomal Domains Replicate with Appropriate Timing

In pre-TDP nuclei, chromosomal domains have not yet been repositioned, and early- and late-replicating domains in vitro are randomly distrubuted with respect to early and late replication domains labeled during the preceding cell cycle in vivo. In post-TDP nuclei, sites of early and late replication in vitro spatially coincide with previously labeled early- and late-replicating domains, respectively.

in origin activity, as the methylation status of sequences at DHFR ori-β correlates with origin activity in hamster cells (references in Gilbert, 1998). Taken together, these observations point to a role for chromatin structure in determining sites of replication initiation in eukaryotes, perhaps by restricting the access of Orc proteins to certain chromosomal sites. The observations that transcriptional control elements are often found near origins and that transcription factors can stimulate origin activity have led to the suggestion that transcription per se may play a role in origin specification. However, the ability of transcription factors to recruit histone deacetylase activity and chromatin remodeling factors is compatible with an indirect role for transcription, mediated by chromatin structure. In contrast to the role of chromatin structure in specifying origin activity suggested by the previous examples, activity of the human β-globin origin does not vary with global changes in chromatin structure in the β-globin gene cluster or β-globin gene transcription in different cell types (Cimbora et al., 2000 and references therein). It is possible that the assays used to analyze locus-wide chromatin structure (nuclease sensitivity and histone acetylation) may not accurately reflect altered states of chromatin in the immediate vicinity of the origin, and further investigation of the structure of chromatin near the β-globin origin in different cell types will be required to resolve this issue. Taken together, these results suggest that chromatin structure can affect origin choice, but this relationship is likely to be complex and may vary from locus to locus.

Replication Timing and Nuclear Repositioning

Chromosomal domains replicate at characteristic times during S phase that often correlate with gene activity: active loci typically replicate early in S phase and inactive loci replicate later. It is not yet clear whether gene activity is influenced by replication timing, or whether replication timing is a consequence of gene activity, but these possibilities are not mutually exclusive. One factor that may influence both gene activity and replication timing is position within the nucleus. The colocalization of inactive genes with Ikaros and HP1 proteins near centromeric heterochromatin in interphase nuclei, and the dynamic repositioning of active versus inactive genes relative to this compartment, suggest that nuclear position and the local protein environment of a chromosomal domain are determinants of gene activity (Francastel et al., 1999 and references therein). Similarly, early- and late-replicating sequences occupy distinct nuclear positions during S phase, suggesting that nuclear position may dictate replication timing. This notion is further supported by the recent demonstration that replication timing of chromosomal domains in CHO nuclei is determined during early G1 at the same time that these domains are repositioned in the nucleus following mitosis. This step, termed the temporal decision point (TDP), is analogous to the origin decision point (ODP) but rather than marking the acquisition of origin specificity, the TDP marks the acquisition of the replication timing program.

The existence of the TDP was demonstrated by an elegant series of experiments using CHO nuclei in *Xenopus* extracts (Dimitrova and Gilbert, 1999). Early-replicating sequences in CHO nuclei are distributed throughout the euchromatic regions of the nucleus, while later-replicating sequences are located at the nuclear periphery and perinucleolar regions; similar patterns are observed in hamster cells and other mammalian cell lines. Gilbert and colleagues isolated CHO nuclei at various points in G1 and compared the distribution of early- and late-replicating domains in the subsequent S phase in *Xenopus* extract (in vitro) to early- and late-replicating domains labeled independently during the previous S phase in hamster cells (in vivo). In nuclei isolated at least 2 hr after metaphase, early- and late-replicating domains are appropriately distributed. In contrast, nuclei isolated only 1 hr after metaphase replicate DNA, but the distribution of early and late replication domains appears random with respect to previously labeled early- and late-replicating sequences (Figure 3). These observations were confirmed by the molecular analysis of specific loci with known replication timing: the proper temporal order of replication was observed only in nuclei isolated after the TDP. Thus, replication timing is programmed in CHO nuclei between 1 and 2 hr after metaphase. Further experiments demonstrate that nuclei that have reached the TDP have not yet acquired the ability to

recognize DHFR ori-β, indicating that the TDP and ODP are independent events. Furthermore, both the ODP and the TDP precede the restriction (R) point, a late G1 control point after which cells are committed to S phase entry independent of growth conditions (Figure 1; references in Gilbert, 1998).

Analysis of cultured CHO cells demonstrates that chromosomal domains reacquire their characteristic distributions in the nucleus 1 to 2 hr after mitosis, precisely the time at which replication timing is programmed (Dimitrova and Gilbert, 1999). This coincidence suggests that nuclear positioning plays a role in replication timing control. Chromosomal repositioning in early G1 has been noted in other cell types, including human fibroblasts in which the position of chromosomal domains correlates with the proliferative state of the cells (Bridger et al., 2000). These observations suggest that altering nuclear position may be a general mechanism for controlling replication in mammals; however, the mechanism by which this occurs is not yet clear. Asymmetric distribution of replication proteins in the nucleus suggests that some locations will favor early replication by allowing the rapid binding or activation of replication factors at the onset of S phase. *Xenopus* and *Drosophila* Orc proteins physically interact with the heterochromatin protein HP1 (Pak et al., 1997), raising the possibility that HP1 may influence replication timing by altering the availability of replication factors. Consistent with this, replication timing of the human β-globin locus is correlated with the position of the locus relative to centromeric heterochromatin in interphase nuclei (Schübeler et al., 2000). A relationship between nuclear position and replication timing is also evident in yeast, and it has been demonstrated that the transcriptional silencing protein Sir3, which forms foci near the nuclear periphery, is required for late replication from a telomeric origin (Stevenson and Gottschling, 1999). Interestingly, the late timing of a yeast telomeric origin is also established during G1 (Raghuraman et al., 1997), and early- and late-replicating yeast origins assume distinct nuclear distributions in G1 phase nuclei (Heun et al., 2001), suggesting that the mechanism that programs replication timing soon after metaphase may be evolutionarily conserved.

Conclusion and Outlook

The existence of G1 steps at which origin choice and replication timing are programmed in mammalian nuclei was demonstrated using a heterologous system based on *Xenopus* extracts. In this system, the origin decision point correlates with ORC assembly on chromatin, and the temporal decision point coincides with the postmitotic repositioning of chromosomal domains in the nucleus. It is likely that these control steps identified in vitro reflect regulatory events in living cells. However, although we do not yet have a complete picture, potential differences among metazoan replication control mechanisms are already apparent. For example, the number and spatial arrangement of replication foci differs between mammalian primary cells and cell lines (Kennedy et al., 2000), and different profiles of Orc protein expression during the cell cycle are evident among metazoans (Natale et al., 2000 and references therein), suggesting different replication control mechanisms may exist. These differences emphasize the need for caution when extrapolating results from one organism or experimental system to another. The identification of further similarities and the reconciliation of differences among these diverse systems will be important for refining models of replication control in higher eukaryotes.

Selected Reading

Abdurashidova, G., Riva, S., Biamonti, G., Giacca, M., and Falaschi, A. (1998). EMBO J. *15*, 2961–2969.

Aladjem, M.I., Rodewald, L.W., Kolman, J.L., and Wahl, G.M. (1998). Science *281*, 1005–1009.

Bridger, J.M., Boyle, S., Kill, I.R., and Bickmore, W.A. (2000). Curr. Biol. *10*, 149–152.

Cimbora, D.M., Schübeler, D., Reik, A., Hamilton, J., Francastel, C., Epner, E.M., and Groudine, M. (2000). Mol. Cell. Biol. *20*, 5581–5591.

Dimitrova, D.S., and Gilbert, D.M. (1998). J. Cell Sci. *111*, 2989–2998.

Dimitrova, D.S., and Gilbert, D.M. (1999). Mol. Cell *4*, 983–993.

Francastel, C., Walters, M.C., Groudine, M., and Martin, D.I.K. (1999). Cell *99*, 259–269.

Gilbert, D.M. (1998). Curr. Opin. Genet. Dev. *8*, 194–199.

Heun, P., Laroche, T., Raghuraman, M.K., and Gasser, S.M. (2001). J. Cell Biol., in press.

Kennedy, B.K., Barbie, D.A., Classon, M., Dyson, N., and Harlow, E. (2000). Genes Dev. *14*, 2855–2868.

Kreitz, S., Ritzi, M., Baack, M., and Knippers, R. (2000). J. Biol. Chem., in press. Published online December 1, 2000. 10.1074/jbc.M009473200.

Lu, Z.H., Sittman, D.B., Romanowski, P., and Leno, G.H. (1998). Mol. Biol. Cell *9*, 1163–1176.

Maric, C., and Hyrien, O. (1998). Chromosoma *107*, 155–165.

Mendez, J., and Stillman, B. (2000). Mol. Cell. Biol. *20*, 8602–8612.

Mizushima, T., Takahashi, N., and Stillman, B. (2000). Genes Dev. *14*, 1631–1641.

Natale, D.A., Li, C.-J., Sun, W.-H., and DePamphilis, M.L. (2000). EMBO J. *19*, 2728–2738.

Pak, D.T., Pflumm, M., Chesnokov, I., Huang, D.W., Kellum, R., Marr, J., Romanowski, P., and Botchan, M.R. (1997). Cell *91*, 311–323.

Raghuraman, M.K., Brewer, B.J., and Fangman, W.L. (1997). Science *276*, 806–809.

Schübeler, D., Francastel, C., Cimbora, D.M., Reik, A., Martin, D.I.K., and Groudine, M. (2000). Genes Dev. *14*, 940–950.

Stevenson, J.B., and Gottschling, D.E. (1999). Genes Dev. *13*, 146–151.

Walter, J., Sun, L., and Newport, J. (1998). Mol. Cell *1*, 519–529.